RED CELLS

Formation of Dense Erythrocytes in SAD Mice Exposed to Chronic Hypoxia: Evaluation of Different Therapeutic Regimens and of a Combination of Oral Clotrimazole and Magnesium Therapies

By Lucia De Franceschi, Carlo Brugnara, Philippe Rouyer-Fessard, Helene Jouault, and Yves Beuzard

We have examined the effect of hydroxyurea (HU), clotrimazole (CLT), magnesium oxide (Mg), and combined CLT+Mg therapies on the erythrocyte characteristics and their response to chronic hypoxia in a transgenic sickle mouse (SAD) model. SAD mice were treated for 21 days with 1 of the following regimens (administered by gavage): control (n = 6), HU (200 mg/d; n = 6), CLT (80 mg/kg/d, n = 5), Mg (1,000 mg/kg/d, n = 5), and CLT+Mg (80 and 1,000 mg/kg/d, respectively, n = 6). Nine normal mice were also treated as controls (n = 3), HU (n = 3), and CLT+Mg (n = 3). Treatment with HU induced a significant increase in mean corpuscular volume and cell K content and a decrease in density in SAD mice. Treatment with the CLT and Mg, either alone or in combination, also increased cell K and reduced density in SAD mice. After 21 days of treatment, the animals were exposed to hypoxia (48 hours at 8% O2) maintaining the same treatment. In the SAD mice, hypoxia induced significant cell dehydration. These hypoxia-induced changes were blunted in either HU- or Mg-treated SAD mice and were completely abolished by either CLT or CLT+Mg treatment, suggesting a major role for the Gardos channel in hypoxia-induced dehydration in vivo.

The availability of an animal model for sickle cell anemia offers a useful tool for studying the pathophysiology of the disease and for evaluating the effectiveness of therapeutic agents in vivo. Several different transgenic mouse models for SS disease are available. Many of these models show (to different degrees) significant RBC sickling upon deoxygenation in vitro and the presence of circulating ISC in vivo. The 2 more recent models seem to mimic closely the clinical and pathologic features of the human disease. The SAD mouse model has been widely used, especially for studies on ion transport and cell dehydration, although these mice do not have anemia, have only mild reticulocytosis, and have normal RBC survival (C. Joiner, personal communication, December 1998). The ion transport pathways of SAD erythrocytes have been characterized in detail and their response to either oral CLT or Mg therapies reproduces that seen in patients with SS disease.

Hydroxyurea (HU) therapy induces macrocytosis, leukopenia, and an increase of the synthesis of the β minor globin chain, with improvement of anemia in a mouse model of human β thalassemia. We have also demonstrated that oral Mg supplementation ameliorates erythrocyte dehydration by increasing erythrocyte Mg and K contents and reducing K-Cl cotransport activity.11,13 We have shown that long-term (6 months) oral Mg supplementation (using Mg pidolate salts) induces a significant reduction in the incidence of acute painful crises in patients with SS disease.14

The Ca2+-activated K transport induces K loss and erythrocyte dehydration when cytosolic free Ca2+ increases, as occurs upon deoxygenation of sickle cells.15 We have shown that treatment with clotrimazole (CLT), a specific inhibitor of the Gardos channel,16,17 can prevent erythrocyte dehydration both in the SAD mouse model and in SS patients.18,19

Although the percentage of circulating dense cells does not predict disease severity, an inverse correlation has been demonstrated between the percentage of irreversibly sickled cells (ISC) and erythrocyte survival. α Thalassemia and an increased cellular content of fetal Hb (Hb F) have been shown to be associated with a reduction in the number of circulating dense cells. Dense cells have also been shown to increase before or in the very first phase of painful crises and to decrease significantly thereafter. Dense ISC have been shown to play an important role in the trapping of cells in postcapillary venules and associated microvascular obstructions.

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thalassemia intermedia. In normal mice, 30 days of HU therapy induce macrocytosis and leukopenia, with no changes in reticulocyte counts. Because there is no clearly demonstrable equivalent of Hb F in mice, studies with HU in SAD mice may be helpful to identify effects that are not related to increased cellular concentration of Hb F. Clinical studies in patients with SS disease have identified cellular changes that are independent of Hb F levels and may explain some of the beneficial effects of HU therapy.

The human and mouse studies indicate that both Gardos channel and K-Cl cotransport are involved in the in vivo generation of dense sickle cells, as recent in vitro studies suggest. The objectives of this study using the SAD mouse model are to determine whether chronic hypoxia (48 hours) induces in vivo changes in erythrocyte features, including the formation of dense cells; what the effects are of different pharmacological regimens, including either HU, CLT, or Mg on the cellular changes induced by hypoxia; and what is the added benefit of combining CLT and Mg therapies.

MATERIALS AND METHODS

**Drugs and chemicals.** NaCl, KCl, ouabain, bumetanide, Tris (hydroxymethyl) aminomethane (Tris), 3(N-morpholino)propanesulfonic acid (MOPS), choline chloride, and Acatoinox were purchased from Sigma Chemical Co (St Louis, MO). MgCl₂, dimethylsulfoxide (DMSO), n-butyl phthalate, and all other chemicals were purchased from Fisher Scientific Co (Fair Lawn, NJ). Microhematocrit tubes were purchased from Drummond Scientific Co (Bromall, PA). All solutions were prepared using double-distilled water.

**Animals and experimental design.** Transgenic HbB₃gamma8thalamus SAD (SAD) mice were used for the experiment, whereas the control group consisted of nontransgenic litter mates. All of the mice were obtained from breeding performed in the animal facility of INSERM at Henri Mondor Hospital (Creteil, France). Males between 4 and 6 months of age (weight, 28 to 30 g) were used for this study. Twenty-eight SAD mice were divided into 5 different groups: control (n = 6), HU (200 mg/d), CLT (80 mg/kg/d), Mg (1,000 mg/kg/d), respectively, and CLT + Mg (80 and 1,000 mg/kg/d, respectively, n = 6).

Nine normal control mice were divided into 3 groups, which were treated for 21 days with 1 of the following regimens: control, HU (200 mg/d), and CLT + Mg (80 and 1,000 mg/kg/d, respectively).

HU was suspended in water (0.2 mL). CLT was suspended in a solution containing deoxycholate (5 mg/mL) and cellulose (0.6%) to a final concentration of 20 mg/mL. Mg supplementation was achieved by dissolving it in water. HU as well as CLT and Mg were administered by mouse feed. The Mg supplement consisted of magnesium hydroxide (hydroxymethyl) aminomethane (Tris), 3(N-morpholino)propanesulfonic acid (MOPS), choline chloride, and Acatoinox were purchased from Sigma Chemical Co (St Louis, MO). MgCl₂, dimethylsulfoxide (DMSO), n-butyl phthalate, and all other chemicals were purchased from Fisher Scientific Co (Fair Lawn, NJ). Microhematocrit tubes were purchased from Drummond Scientific Co (Bromall, PA). All solutions were prepared using double-distilled water.

**Results**

Effects of HU, CLT, Mg, and CLT + Mg treatments on hematological parameters. HU therapy in normal control mice produced no significant changes in Hct and Hb (data not shown). In SAD mice, HU induced an increase in Hct from 44.4% ± 1.1% to 46.7% ± 1.1%, P < .005, mean corpuscular volume (MCV; from 43.1 ± 0.4 fl to 45.8 ± 0.3 fl, P < .05), Hb, and reticulocyte counts (Table 1) over their normal baseline values and a decrease in WBC counts (Table 1). A shift in the phthalate density distribution curve towards lower values was also observed (Fig 1B and Table 2).

CLT treatment of SAD mice resulted in a significant increase in Hct (from 43.9% ± 1.3% to 47.0% ± 1.2%, P < .05) and a decrease in cell density (Table 2 and Fig 1C). Hb, reticulocyte, and WBC counts were unchanged after CLT therapy (Table 1). Mg treatment of SAD mice resulted in significant increases in Hct (from 43.6% ± 0.7% to 45.5% ± 0.2%, P < .05) and Hb (Table 1) and decreased cell density (Table 2 and Fig 1D), as described in our previous report. Mg treatment did not induce significant changes in either reticulocyte or WBC counts (Table 1).

A combination of CLT + Mg treatments in normal control mice resulted in significant increases in Hb (from 14.1 ± 0.4 to 15.3 ± 0.3 g/dL, P < .02) and Hct (from 45.8% ± 1.3% to 48.2% ± 0.6%, P < .02). Because we have previously demonstrated that CLT administration did not affect the hematological parameters of normal mice, whereas Mg increased Hb...
Table 1. Effects of HU, CLT, Mg, and CLT + Mg treatments under ambient and hypoxic conditions on hematological parameters in SAD mice

<table>
<thead>
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<th>Baseline (n = 5)</th>
<th>21 days</th>
<th>Hypoxia (n = 5)</th>
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<tr>
<td></td>
<td>Hb (g/dL)</td>
<td>WBC (×10^9/L)</td>
<td>Hb (g/dL)</td>
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<tr>
<td>HU</td>
<td>12.5 ± 0.2</td>
<td>12.8 ± 0.6</td>
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<td>CLT</td>
<td>12.4 ± 0.3</td>
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<tr>
<td>Mg</td>
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<td>12.1 ± 0.6</td>
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<td>CLT + Mg</td>
<td>12.6 ± 0.4</td>
<td>12.3 ± 0.3</td>
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Data are presented as the means ± SD. *P < .05 compared with baseline. **P < .01 for comparison between after 21 days and hypoxia.

HU treatment did not modify the activity of the Gardos channel in either normal control (data not shown) or SAD mice (Table 2). The erythrocyte K content was unchanged by HU treatment in normal mice (data not shown), whereas it increased significantly in SAD mice (Table 2).

CLT treatment of SAD mice induced marked inhibition of the Gardos channel and significantly increased erythrocyte K content (Table 2), as described in our previous study.18

Mg treatment of SAD mice yielded no changes in Gardos channel activity and significantly increased erythrocyte K content (Table 2), as shown before.11 Erythrocyte Mg content increased from 9.6 ± 0.7 mmol/kg Hb to 13.9 ± 1.1 mmol/kg Hb (n = 5, P < .05) and plasma Mg increased from 0.94 ± 0.9 mmol/L to 1.5 ± 0.7 mmol/L (n = 5, P < .05).

Combined CLT + Mg treatments of normal control mice inhibited Gardos channel without affecting K content (data not shown). As expected, SAD mice treated with CLT + Mg showed a reduction in the activity of the Gardos channel and increased erythrocyte K content (Table 2). CLT + Mg treatment resulted in an increase in both plasma and erythrocyte Mg levels in SAD mice (plasma: 0.75 ± 0.4 mmol/L at baseline v 1.7 ± 0.6 mmol/L, n = 5, P < .05; erythrocytes: 8.7 ± 1.2 mmol/kg Hb at baseline v 12.9 ± 1.7 mmol/kg Hb after treatment, n = 5, P < .05).

Effects of hypoxia. To evaluate the effect of the 4 therapeutic regimens on the changes induced by hypoxia, control and transgenic mice were exposed for 48 hours to an atmosphere containing 8% O2. No significant changes in Hct or Hb were observed in normal control mice after hypoxia (data not shown).

In untreated SAD mice, hypoxia induced a shift of the phthalate density profiles toward higher erythrocyte density values, indicating that hypoxia exacerbates RBC dehydration (Table 2 and Fig 1A). Erythrocyte K content also decreased significantly with hypoxia (Table 2). No significant changes were observed in either reticulocyte or WBC counts after hypoxia (Table 2).

In HU-treated SAD mice, hypoxia decreased Hb levels (Table 1), MCV (from 45.8 ± 0.3 fL to 43.4 ± 0.2 fL, P < .05), and cell K content (Table 2), whereas cell density showed a trend toward higher values (Fig 1B), which, however, was not statistically significant (Table 2). Reticulocyte or WBC counts did not change with hypoxia in HU-treated SAD mice (Table 1). Cell K content and density in HU-treated SAD mice exposed to hypoxia were still significantly different from those of untreated, hypoxic SAD mice (P < .02 and P < .05, respectively, ANOVA).
In CLT-treated mice exposed to hypoxia, discrepant results were obtained between measured cell density, which increased significantly (Table 2 and Fig 1C), and measured cell K content, which was unchanged (Table 2). This unexplained discrepancy does not allow us to determine with certainty how much of the erythrocyte dehydration induced by chronic hypoxia is mediated by the Gardos channel. It should be noted that, with hypoxia, erythrocyte density and cation contents of CLT-treated SAD mice were still significantly different from those of untreated SAD mice (Table 2, ANOVA, \(P < .005\)), indicating an effect of CLT on hypoxia-induced dehydration.

In Mg-treated mice exposed to hypoxia, a reduction in Hb (Table 1) and cell K content and an increase in cell density were noted (Table 2 and Fig 1D) that almost completely abolished the changes induced by 21 days of Mg therapy. The K loss induced by chronic hypoxia was essentially the same as that of untreated SAD mice, indicating that the K-Cl cotransport plays a minor role in hypoxia-induced dehydration of SAD erythrocytes.
which seems to be mostly a Gardos phenomenon. Interestingly, the density and cation content of Mg-treated mice after hypoxia were still significantly different (P < .005 and P < .03, respectively, ANOVA) than those of hypoxic, untreated SAD mice (Table 2).

In SAD mice treated with CLT+Mg, hypoxia induced no significant changes in either Hb, reticulocyte, or WBC counts (Table 1). Erythrocyte K content and cell density did not change significantly with hypoxia and remained significantly different from baseline values (Table 2) and from untreated SAD mice (P < .002 and P < .005, respectively, ANOVA, Table 2). These data indicate that CLT+Mg treatment almost completely abolished the density changes induced by chronic hypoxia. Because Mg was ineffective in preventing hypoxia-induced dehydration, it is likely that blockade of the Gardos channel is responsible for these effects. However, due to the discrepancies observed in the CLT-treated group and differences in baseline density among the various groups, the superiority of CLT+Mg treatment compared with the other regimens cannot be convincingly demonstrated.

**DISCUSSION**

We have examined in this study the effect of 4 therapeutic regimens, including either HU, CLT, Mg, or CLT+Mg, on the changes induced by a short-term (48 hours) exposure to hypoxia in the SAD mouse model. These studies were prompted by several in vitro and in vivo studies that have identified a role for the erythrocyte Gardos channel and K-Cl cotransporter in promoting erythrocyte dehydration. Combination treatment with CLT and Mg offers the theoretical possibility of interfering with the dehydration of both reticulocytes and mature erythrocytes by inhibiting the 2 major pathways for sickle cell dehydration.

The SAD mouse has shown to be extremely valuable in assessing the cellular effects of therapies aimed at preventing sickle cell dehydration. SAD mouse erythrocytes resemble human sickle erythrocytes in having a reduced K content, normal Gardos channel activity at baseline, and increased K-Cl cotransport. The response observed in SAD mice to either CLT or Mg therapies is similar to that observed in patients with sickle cell disease. Thus, although SAD mice are not anemic, they exhibit significant RBC dehydration and organ damage and are a valuable model for studies on ion transport and blockade of cell dehydration.

K-Cl cotransport plays a major role in the dehydration of sickle erythrocytes and reticulocytes. Transferrin receptor-positive (Tfr⁺) dense reticulocytes have greater K-Cl cotransport activity than Tfr⁻ light reticulocytes, suggesting that K-Cl cotransport may mediate dehydration of young sickle cells. K-Cl cotransport activity is modulated by the erythrocyte Mg content, which is markedly reduced both in transgenic SAD mouse and human sickle erythrocytes. We have shown that oral Mg supplementation induces an increase in RBC Mg content that, in turn, leads to a reduction in K-Cl cotransport activity and cell dehydration. However, although the Gardos channel has been shown to become active with deoxygenation, the role of K-Cl cotransport in promoting dehydration in conditions of hypoxia is not well established. For these reasons, we have examined the effect of pharmacological blockade of these ion pathways in the SAD mouse under conditions of chronic hypoxia.

The results presented here indicate that (1) hypoxia induces formation of dense erythrocytes in SAD mice; (2) HU, CLT, Mg, or CLT+Mg therapies improve the hydration state of erythrocytes and blunt the erythrocyte dehydration induced by hypoxia; (3) hypoxia-induced dehydration in the SAD mouse is mediated almost exclusively by the Gardos channel; and (4) combination of CLT and Mg treatments may have an additive effect in protecting from the erythrocyte dehydration induced by hypoxia, but the results presented here are not unequivocal.

Although other studies have demonstrated formation of dense cells by hypoxia in transgenic sickle mice, no information was available on the mechanisms underlying the formation of dense mouse erythrocytes. Rubin et al. using a mouse model
expressing both human \( \alpha \) and \( \beta^S \) \(^\text{Antilles} \) (50% of total Hb), exposed the transgenic mice for 10 days at 8.4% \( \text{O}_2 \), and showed a significant increase in irreversibly sickled cells. Similar results were obtained by Fabry et al., \(^2\) who exposed the human \( \alpha^S \) and \( \beta^S \) \((\beta^\text{MDD})\) transgenic mice for 3 or 5 days to hypoxia (8% \( \text{O}_2 \)). This group also observed a significant reduction in urine osmolality due to compromised renal function. \(^2\) Reilly et al.\(^3\) examined 3 lines of transgenic Hb S mice with human \( \beta^S \) contents of approximately 30%, 50%, and 80% relative to mouse \( \beta \) globins. Exposure to hypoxia (7% \( \text{O}_2 \)) for 7 days resulted in increased Hct, Hb, and MCV and significant reticulocytosis, indicating that this level of chronic hypoxia significantly stimulated erythropoiesis. An increase in the percentage of cells residing in the most dense fraction was also noted. \(^3\)

Duration of exposure to hypoxia seems to be a critical variable for these studies. We have observed significant reticulocytosis both in control and \( \beta \) thalassemic mice after 5 days of hypoxic exposition (Y. Beuzard, unpublished data). The shorter period of hypoxia (48 hours) allowed us to study erythrocyte changes primarily due to polymerization of Hb SAD. With our study, we were able to evaluate the effect of 4 different treatments on these erythrocyte changes with no significant changes in reticulocyte counts. Recently, hypoxia has been shown to enhance sickle cell adhesion to both macrovascular and human microvascular endothelial cells via the adhesive receptor vascular cell adhesion molecule-1 (VCAM-1), suggesting that reticulocytes may be involved in the enhanced adherence to the hypoxic endothelium. \(^4,5\) It will be of interest to determine whether adhesion to endothelium of sickle transgenic erythrocytes, \(^5\) in addition to being modulated by hypoxia, can also be affected by either HU, CLT, or Mg therapies.

In this study, HU treatment of SAD mice induced a significant increase in MCV and a decrease in WBC counts (Table 1), as observed in humans. However, because mice do not produce Hb F, the effects of HU on erythrocyte cation content and density of SAD mice are not easily explained. Our data clearly indicate that HU has no effect on the activity of the Gardos channel (Table 2). In addition, HU induced a significant reticulocytosis in SAD mice, whereas it usually decreases reticulocyte counts in SS patients. \(^6,7\) We have described a marked reduction of the in vitro adherence of human sickle erythrocytes to endothelium in the early phase of HU therapy. \(^8\) Whether this effect is present in transgenic sickle mice remains to be determined.

These studies provide experimental evidence for a major role of the Gardos channel in promoting dehydration of SAD mice erythrocytes under conditions of chronic hypoxia. They also demonstrate the beneficial effects of HU, CLT, Mg, and CLT+Mg therapies in preventing or blunting the hypoxia-induced dehydration. Combination therapy with CLT and Mg could in theory be superior to single-agent therapy in preventing cell dehydration. However, under experimental conditions that maximize dehydration via the Gardos channel, this potential additive benefit could not be confirmed.

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